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Ceramides Are Bound to Structural Proteins of the Human Foreskin Epidermal Cornified Cell Envelope

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Lyuben N. Marekov and Peter M. Steinert†

From the Laboratory of Skin Biology, NIAMS, National Institutes of Health, Bethesda, Maryland 20892-2752

An important component of barrier function in human epidermis is contributed by ceramides that are bound by ester linkages to undefined proteins of the cornified cell envelope (CE). In this paper, we have examined the protein targets for the ceramide attachment. By partial saponification of isolated foreskin epidermal CEs followed by limited proteolysis, we have recovered several lipopeptides. Biochemical and mass spectroscopic characterization revealed that all contained near stoichiometric amounts of ceramides of masses ranging from about 690 to 890 atomic mass units, of which six quantitatively major species were common. The array of ceramides was similar to that obtained from pig skin, the composition of which is known, thereby providing strong indirect data for their fatty acid and sphingosine compositions. The recovered peptides accounted for about 20% of the total foreskin CE ceramides. By amino acid sequencing, about 35% of the peptides were derived from ancestral glutamine-glutamate-rich regions of involucrin, an important CE structural protein. Another 18% derived from rod domain sequences of periplakin and envoplakin, which are also known or suspected CE proteins. Other peptides were too short for unequivocal identification. Together, these data indicate that involucrin, envoplakin, periplakin, and possibly other structural proteins serve as substrates for the attachment of ceramides by ester linkages to the CE for barrier function in human epidermis.

Mammalian epidermis lies at the interface with the environment, where it plays an essential role in providing a physical, chemical, and water barrier for the organism (1-4). Cornified keratinocytes, which constitute the major cell type of the epidermis, have evolved an elaborate barrier system. Part of this is contributed by the cornified cell envelope (CE),¹ which is a 15-20-nm thick layer on the periphery of the corneocyte, and consists of two components. An ~15-nm-thick layer of several defined structural proteins is deposited on the intracellular surface of the cell membrane in the upper spinous and granular cells of the living epidermis (5-8). These proteins become cross-linked together by disulfide bonds and $N^1(\gamma\text{-glutamyl})\text{lysine}$ or $N^1N^2\text{-bis}(\gamma\text{-glutamyl})\text{sperrmidine}$ isopeptide bonds formed by the action of transglutaminases (5-9). This process appears to begin with the cross-linking of certain early protein components, such as involucrin and envoplakins, at or near to the site

of desmosomes (10-13), which together form a scaffold (12, 14) for the subsequent stages of addition of elafin, small proline-rich proteins, and much larger amounts of loricrin (10-12, 15-17). At a late stage of protein envelope assembly, perhaps in upper granular cells, the lipid envelope component is assembled (reviewed in Refs. 2-4). The phospholipid-rich cellular plasma membrane is rebuilt as a 5-nm-thick layer of ceramide lipids, which subsequently become covalently attached to the protein envelope on the extracellular surface (18-21). The lipids are synthesized, packaged into lamellar bodies, and extruded from the granular cells into the intercellular space (reviewed in Refs. 2-4 and 22). A minor but important component are the acylglucosyl-ceramides, of which about two-thirds are converted to ω -hydroxyceramides that become covalently attached by ester linkages to the protein envelope to complete CE assembly. Many other lipids, such as cholesterol esters and free fatty acids, function to form the lamellae in the intercellular milieu and contribute in a major way to the barrier against desiccation (2-4, 17-23). One idea is that the ceramides that are ester-linked to the outer surface of the CE contribute a hydrophobic surface to the corneocyte that has important consequences for water barrier function by associations with and perhaps organization of intercellular lipids (2).

We have been interested in the structure of the CE and have devised models on the nature and sequences of the proteins involved in its formation (10-12). However, rather less is known about how the lipid envelope becomes attached to the protein envelope. Specifically, although a model has been suggested (2, 21, 24), the nature of the protein substrate for lipid addition is not yet known. In this study, we explored this question. Partial removal of ester-linked lipids from isolated foreskin epidermal CEs by saponification has allowed the isolation of lipopeptides consisting of ceramides attached to certain glutamines and/or glutamic acid residues of involucrin, as well as other likely CE proteins.

MATERIALS AND METHODS

Preparation of CEs—“Mature” CEs were prepared from the stratum corneum tissue of freshly-prepared foreskin epidermis as described previously (10-12). They were resuspended in a buffer of 0.1 M *N*-ethylmorpholine acetate (pH 8.3) and digested with trypsin (Sigma, sequencing grade, 1% by weight, for 2 h at 37 °C) to remove contaminating adherent proteins (12) and pelleted at 14,000 $\times g$.

Saponification—Aliquots of CEs containing known amounts of protein were resuspended in 1 M KOH in 95% methanol for 15-120 min at 45 °C, washed with methanol, and dried. Potential ceramide-peptide adducts were reacted similarly to hydrolyze the ester linkages (generally a 2-h reaction). The saponified lipids were recovered by extraction into chloroform/methanol (1:2 v/v) and dried.

Protein Chemistry Procedures—Amino acid analysis of hydrolyzed samples (5.7 M HCl at 110 °C for 24 h *in vacuo*) was used to routinely measure protein amounts. Partially or completely saponified CEs were resuspended (1 mg of CE protein/ml) in buffer and redigested with trypsin or proteinase K (Life Technologies, Inc.; 1% by weight) for up to 3 h at 37 °C. The solubilized material was collected following removal of the CE remnants by pelleting and dried. Peptides were fractionated on a C₁₈ high performance liquid chromatography (HPLC) column as be-

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† To whom correspondence should be addressed: Bldg. 6, Rm. 425, NIAMS, National Institutes of Health, Bethesda, MD 20892-2752. Tel.: 301-496-1578; Fax: 301-402-2886; E-mail: pemast@helix.nih.gov.

¹ The abbreviations used are: CE, cornified cell envelope; HPLC, high performance liquid chromatography.

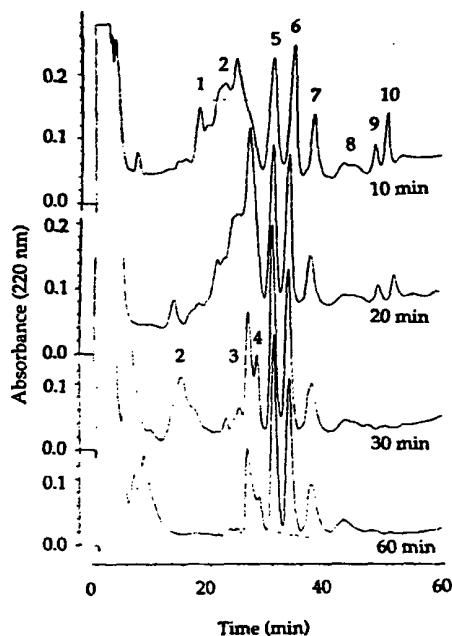


FIG. 1. Separation by HPLC on a C_4 column of proteinase K peptides following 15 min of partial saponification. The profiles shown denote different times of proteinase K digestion. The 10 arbitrarily numbered peaks of material used for further analyses are indicated.

fore (10, 11). To recover potential lipopeptide adducts, a Phenomenex C_4 HPLC column (200×2.5 mm) was used at a flow rate of 0.22 ml/min with 10% acetonitrile containing 0.08% trifluoroacetic acid and with a linear gradient of up to 15% (v/v) isopropanol in acetonitrile and collected into 0.11 -ml fractions. Aliquots of selected peptide peaks were subjected to amino acid analysis or attached to a solid support (Sequenator-AA, Millipore) for sequencing as described previously (11, 12). In addition, aliquots (5 – 20 pmol) were removed for mass measurements by mass spectroscopy both before and after saponification, using a matrix assisted laser desorption ionization procedure. Following an initial scan from 600 atomic mass units (below which reliable data could not be obtained), scans were repeated in the vicinity of the major mass species.

Ceramide Assay—Aliquots of potential lipopeptides were saponified for 2 h as described above. The released ceramides were then extracted with chloroform/methanol (95:5) and quantified using the diacylglycerol assay reagent kit and $[\gamma-^{32}P]ATP$ (Amersham Pharmacia Biotech) as described (25). This reaction specifically labels an hydroxyl of the sphingosine moiety of ceramide only. High performance thin layer chromatography silica gel plates (Merck) were developed with chloroform:acetone:methanol:acetic acid:water $10:4:3:2:1$. N -stearoyl-D-sphingosine (C_{18} ceramide) (Sigma) was used as quantitation standard. The dried plates were then exposed for up to 6 days. Bands were quantitated by scanning densitometry of several exposures of the x-ray films.

RESULTS AND DISCUSSION

Recovery of Potential Lipopeptides from Foreskin Epidermal CEs—Previously, we have demonstrated that saponification of foreskin epidermal CEs, a mild alkaline hydrolysis procedure that is likely to cleave ester bonds, exposes for proteolytic attack a number of structural proteins of the protein portion of the CE (11, 12). Our immunogold electron microscopy analyses and proteolytic digestion experiments suggested that in intact CEs, the ester-linked lipids mask access to structural proteins corresponding to the inner or earlier stages of CE assembly (12). These proteins were predominantly involucrin, envelopeplakin, and desmoplakin (12), but they may also consist of other desmosomal and calcium-binding proteins (12, 13). In this paper, we have addressed the questions of the nature of the possible protein substrates to which these lipids are covalently attached, their sites of attachment, and the properties of the

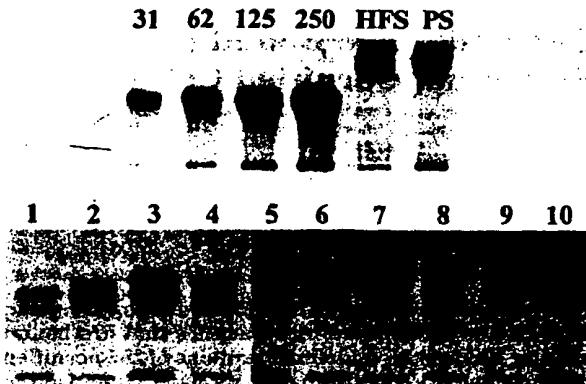


FIG. 2. Ceramide assay using the diacylglycerol reaction. *Upper panel*, autoradiographs of reactions with the C_{18} standard (amounts shown), as well as ~ 50 pmol of total ceramides recovered from human foreskin epidermis (HFS) or pig skin (PS). *Lower panel*, autoradiographs of reactions with ~ 10 pmol of each of the 10 peaks of Fig. 1. Multiple exposures were developed in order to quantitate the amounts of ceramide in relation to the C_{18} standard. Note that in many peaks, the labeled products migrated faster than the standard but at rates similar to those of HFS and PS, suggestive of the presence of longer ceramides.

TABLE I
Sequences of peptides resolved on C_4 column of Fig. 1
Single letter amino acid code is used. The letters in boldface delineate the possible site of attachment of ester-linked ceramides.

Peak number	Sequence	Calculated mass atomic mass units	Comment
1	Complex		
2	Complex		
3	PEQECEQQEK	1246	Involucrin-like, residues 70–79 Glu^{76} is a Gln in involucrin
4	EEEKKL	775	Involucrin residues 123–128
5	QQEHW ^a	725	Involucrin-like, residues 87–91 Glu^{89} is a Gln in involucrin
6	EEEGQL	833	Involucrin, residues 386–392
7	NTHQEQQMK	886	Involucrin, residues 27–33
8a	DQELNKQ	872	Involucrin-like, residues 115–121 Glu^{117} is a Gln in involucrin
8b	DQELDQEL	986	Involucrin-like, residues 130–137 Glu^{132} is a Gln in involucrin
9	Complex		
10	Complex		

^a As determined from databases, this sequence occurs only in a *Drosophila* protein.

attached lipids. We reasoned that by reducing the time of an initial saponification reaction to only a few minutes, followed by limited proteolysis, it should be possible to isolate peptides with ester-linked lipid adducts. Such adducts could be identified by HPLC fractionation on a C_4 HPLC column monitored at 220 nm. Free hydrophilic peptides typically found in CE proteins should not be retarded, and retarded free lipids cannot be detected at 220 nm. We performed a number of trial experiments titrating the degree of saponification (in the range of 10 – 30 min) and subsequent proteolysis with trypsin or proteinase K singly or in combination. Although trypsin released sev-

FIG. 3. Separation by HPLC on a C_{18} column of unresolved peptides of peaks 1 and 2 of Fig. 1 following saponification. Each peak resolved after 10 min was subjected to sequencing, and those with three or more residues are listed in Table II.

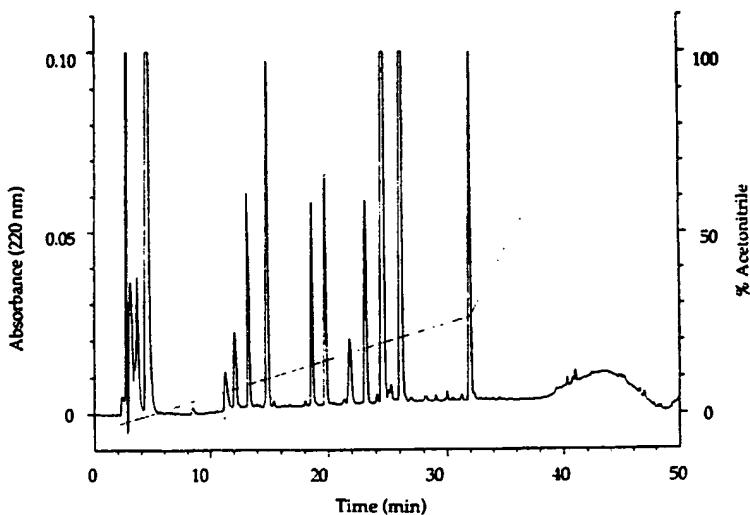


TABLE II
Sequences of short peptides resolved on C_{18} column of Fig. 3

Peak elution time	Amount	Calculated mass	Sequence	Comment
min	%	atomic mass units		
11.6	1		EQQ	
12.3	1		LGE	
13.7	2	961	EQEEAAEAR	Exact match only with plant proteins and human periplakin residues 1430–1437
14.9	4		VEEA	VQEA sequence is present in human desmoplakin (residues 1359–1362) and human envoplakin (residues 655–658)
18.3	2	746	EQQTLQ	Exact match only with plant proteins, but human envoplakin-like, residues 1607–1612; Glu ¹⁶⁰⁷ is a Gln in envoplakin
19.8	2		ELEE	
22.0	1		EQQ	
23.6	2		EESS	
24.5	7		EEEE	
26.1	6	886	EQQLLQQ	Exact match only with plant proteins, but human envoplakin-like, residues 871–877; Glu ⁸⁷¹ is a Gln in envoplakin
32.1	4	701	EELEAL	Exact match only with plant proteins and with human periplakin, residues 968–973

eral retarded peaks resolvable on the C_4 column, a reproducibly better yield of 10 arbitrarily numbered peaks was obtained by proteinase K digestion alone (Fig. 1), even though a significant portion of the peptide material released would have originated from the cytoplasmic (loricrin-rich) face of the CE, where lipids are largely absent (10–12). Optimized conditions were obtained for 15 min of saponification and 10–60 min of proteinase K digestion. Of these, peaks 5–7 were stable for ~1 h of digestion, peaks 3 and 4 became well resolved only after 20–30 min of digestion, peaks 8–10 were lost after ~20 min of digestion, and peaks 1 and 2 were rapidly lost in a time- and shape-dependent manner. The 15-min saponification procedure alone did not result in a significant release of peptide material. In a typical experiment with 15 min of saponification followed by digestion with proteinase K, we released 5.1% (10 min), 12.7% (30 min), 15.8% (1 h) and 19.5% (3 h, complete digestion, cf. 29% in Ref. 11) of the protein mass of the CEs used. Of this, the total amount of retarded peptide material recovered in Fig. 1 represents ~0.4% (10-min digestion) or 0.25% (60-min digestion) of the 220-nm absorbing material eluted from the HPLC column, which corresponds to 0.02–0.05% of the total protein mass of the CEs, or ~250 ng/mg of CEs. None of the retarded peptide fractions contained the isodipeptide cross-link, indicating that

different regions of CE proteins were used for lipid attachments and for cross-linking.

The material from each peak was recovered from the pooled epidermis of 20–30 foreskins. By amino acid analysis, each contained protein, and based on the composition of the simplest peptides (see below), the yields were 20–100 pmol. After a 2-h resaponification reaction, followed by chromatography on the same HPLC column, the 220-nm absorbing peptides of each peak eluted at the column wash, and there was no detectable retarded material. These data suggest the presence of ester-linked hydrophobic lipopeptides.

Retarded Peptide Peaks Contain Near-Stoichiometric Amounts of Ceramides—Earlier data have demonstrated that a major class of lipid molecules ester-linked to the CE proteins are ceramides (19–24). To ascertain whether the retarded peptide-containing species identified in Fig. 1 contained ceramides, we used an established method that specifically incorporates a phosphate group onto an hydroxyl group of the sphingosine moiety of a ceramide (25). Using 10–40-pmol aliquots (based on peptide content), the data of Fig. 2 demonstrate that each peak contains ceramides. Following densitometric scans of the bands, and in relation to the reaction with the C_{18} ceramide standard, we calculated that the peaks contain 0.6

A

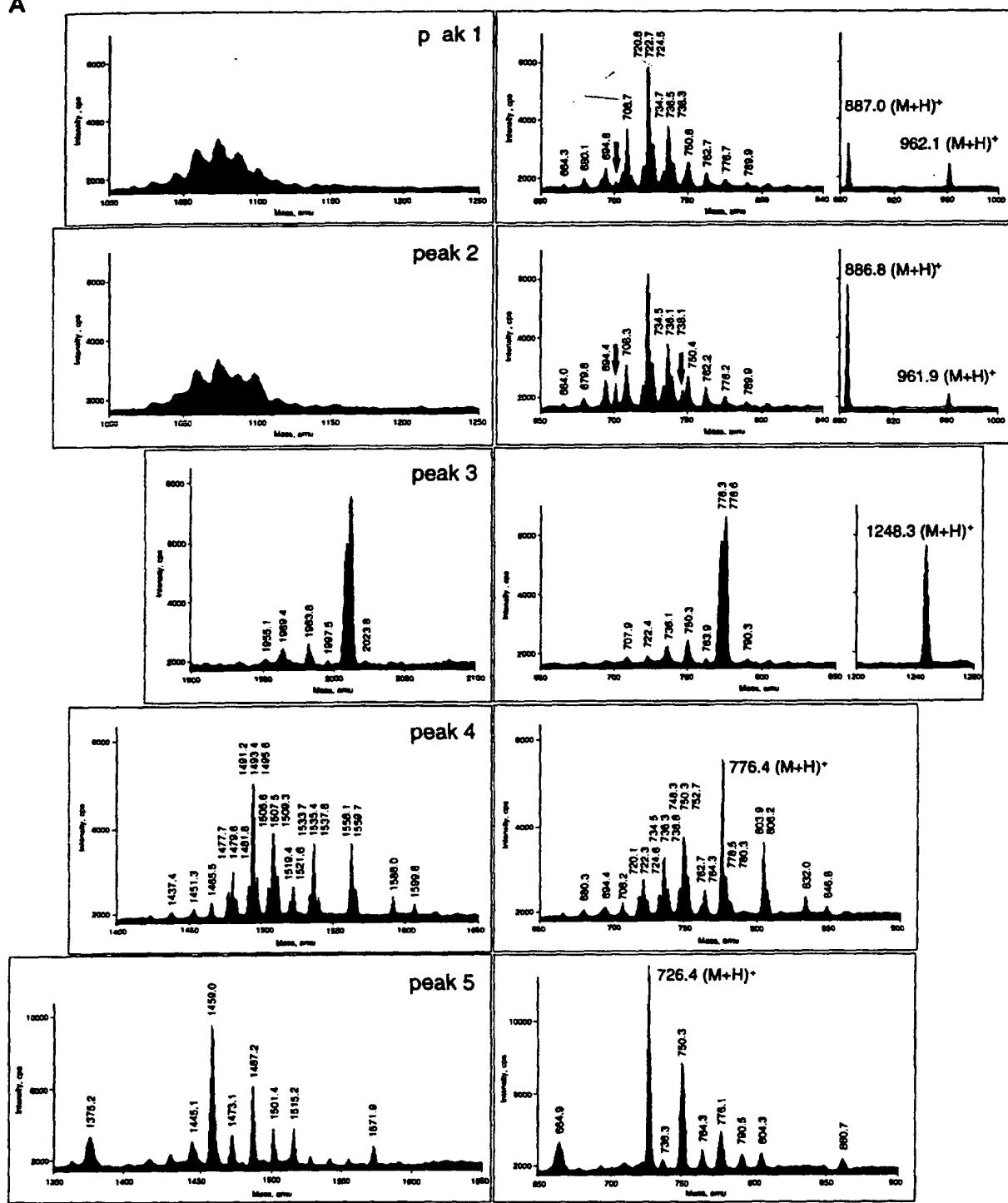


FIG. 4. Mass spectroscopy of peaks before (left panels) and after (right panels) saponification. Following initial scans to identify the size ranges of significant masses, spectra were performed over narrower mass ranges to generate these deconvoluted spectra. Data for peak 9 are not shown. The masses of ceramide-peptide (left) or ceramide (right) peaks are indicated by the vertical annotations. The masses of likely peptide species are indicated by horizontal annotations. For peaks 1 and 2, the arrows (right) indicate masses of 701 and 746 atomic mass units, which correspond to the masses of the peptides EELEAL and EQQTLQ (see Table II). amu, atomic mass units.

mol (peaks 4 and 10), 0.7 mol (peak 2), 0.8 mol (peaks 1, 5, 6, and 9), or up to 0.9 mol (peaks 3, 7, and 8) of ceramide/mol of peptide. The somewhat lower stoichiometry and the multiplicity

of species may indicate degradation or oxidation of the ceramides. Alternatively, because most peptide peaks contained multiple ceramide bands that migrated significantly

B

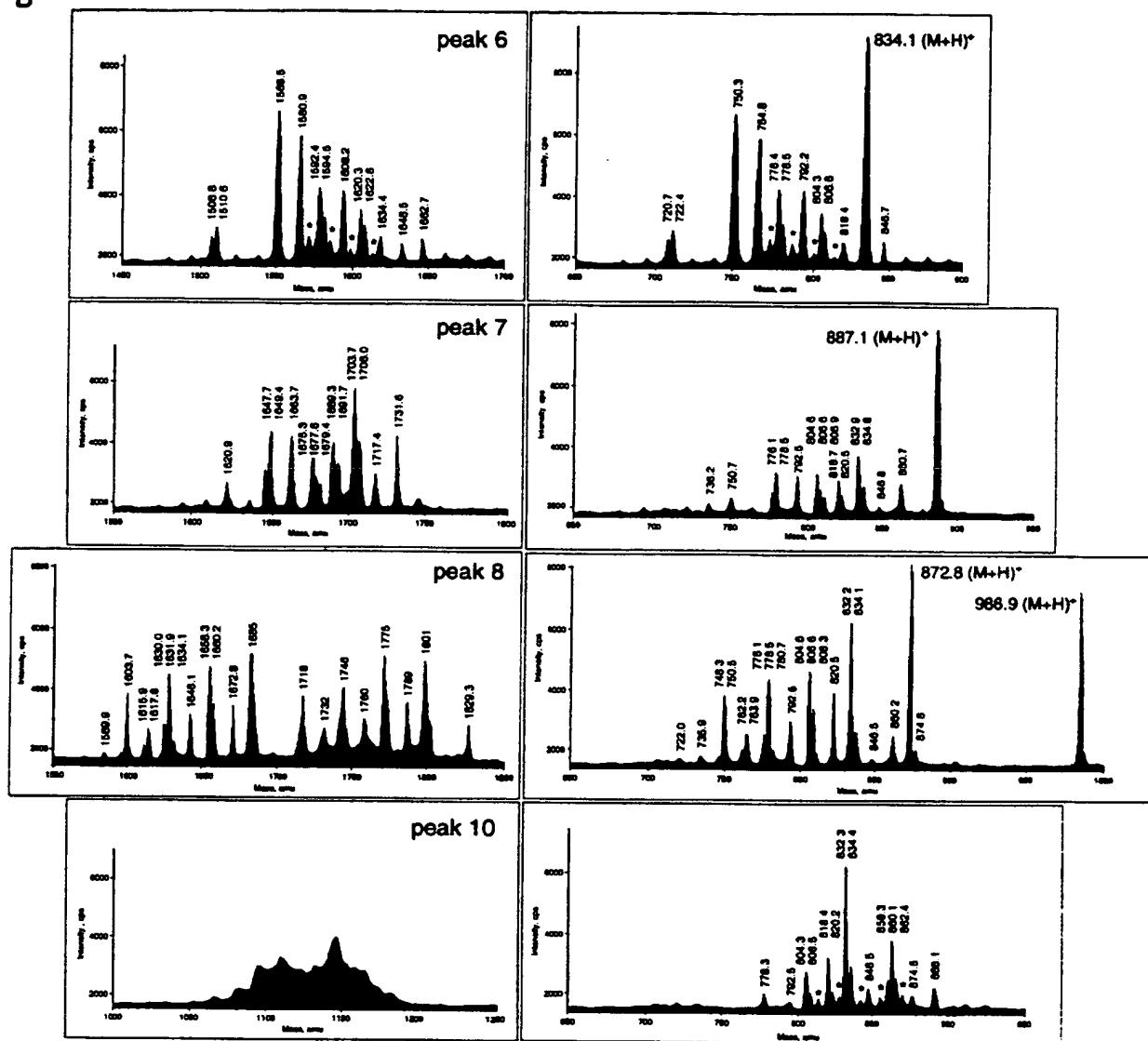


FIG. 4—continued

faster than the C_{18} ceramide standard (Fig. 2), the bands may represent species with variably-sized sphingoid and/or fatty acid chains that were considerably longer than those of the C_{18} ceramide standard. Fig. 2 also shows the reaction of 50 pmol of ceramides recovered from a 2-h saponification reaction of human foreskin CEs and pig skin CEs (a kind gift from Dr. P. Wertz). These broad bands suggest the presence of multiple unresolved ceramide species of similar size to those of the 10 recovered peaks.

Following a 2-h saponification reaction, we calculated that the total yield of human foreskin CE ceramides was 2750 pmol/mg of CE protein. Assuming that the CE is about 15 nm thick and its density is about 1 g/cm^3 (26), 1 mg of protein would occupy about $7 \times 10^{16} \text{ nm}^2$ of CE surface. This means that there is about 1 molecule of ceramide/40 nm 2 of CE. Based on the predicted size of these ceramides (Refs. 19–21 and see below), this means that the entire CE surface is effectively coated by a monomolecular layer of ceramides (19, 20).

Finally, we could account for 15–20% of this total amount of

ceramides in separate experiments by summation of the yields recovered from the 10 peaks of Fig. 1. Another ~70% of the total ceramides was present in the CE pellet following the 15-min limited saponification and 60-min proteinase K digestion procedures.

Six Retarded Peaks Contain Identifiable Involucrin or Involucrin-like Sequences—Aliquots (5–20 pmol) of each peak were used for amino acid sequencing. As Table I shows, peaks 3–8 contained peptides of sequences that are either identical (peaks 4, 6, and 8) or very similar (peaks 3, 5, and 7) to human involucrin. In peaks 3, 5, and 7, a Gln residue in human involucrin was sequenced as a Glu residue. There are two possible explanations for this. The first is that these Gln residues may have participated in the initial step of a cross-linking reaction with transglutaminases but failed to complete the transfer of the enzyme-substrate complex to an acceptor substrate amine, such as a Lys residue: the net result would be deamidation of the Gln residue (27–29). We think that this is a relatively rare event because we have found only a few cases of

TABLE III
Relative amounts of ceramides

The quantitatively major species are shown in bold (see also Table IV).

Mass	Peak number									
	1	2	3	4	5	6	7	8	9	10
atomic mass units										
664	0.01	0.01				0.13				
680	0.01	0.01		0.01						
694	0.03	0.04		0.01						
706	0.04									
708	0.17	0.12	0.02	0.02						
710	0.03									
720	0.07	0.06		0.03		0.03				
722	0.25	0.27	0.02	0.05		0.04		0.01		
724	0.11	0.10		0.03						
734	0.04	0.06		0.04						
736	0.13	0.11	0.05	0.12	0.03		0.02	0.02		
738	0.05	0.08		0.06						
748				0.05					0.01	
750	0.05	0.07	0.06	0.17	0.47	0.26	0.04	0.10	0.05	0.01
752				0.06						
762	0.02	0.04		0.03				0.02		
764			0.01	0.04	0.05	0.18		0.05		
776	0.01	0.02	0.39		0.18	0.10	0.07	0.04	0.06	
778	0.01		0.46	0.06		0.08	0.10	0.12	0.14	0.02
780				0.03				0.03	0.05	
790		0.01			0.05					
792						0.09	0.10	0.05	0.03	0.01
804				0.10	0.05	0.06	0.11	0.09	0.15	0.06
806				0.06		0.04	0.06	0.06	0.10	0.04
808							0.05	0.01	0.04	
818						0.03	0.09		0.06	0.07
820							0.05	0.08	0.04	0.04
832				0.02			0.16	0.19	0.12	0.34
834							0.08	0.05	0.04	0.07
846				0.01		0.02	0.01	0.01	0.02	0.03
858									0.05	
860				0.03			0.06	0.05	0.04	0.11
862										0.05
874								0.01	0.01	0.01
888								0.01	0.02	

such modifications in the sequences of many cross-linked peptides characterized to date.² Moreover, such hydrophilic peptides would not have been retarded by the C₄ column. A second, more plausible possibility is that a lipid adduct was linked through an ester bond to the Gln residue, which, following hydrolysis (saponification) of the ester bond, would generate a Glu residue. Such an hydrolysis would likely occur during the amino acid sequencing chemistry reactions. Thus, these modified residues identify the likely target position of lipid attachment. However, in peptides 4, 6, and 7, in which there was an exact match with involucrin, the lipid was likely attached to an existing Glu residue, but the residue position of modification could not be ascertained.

In total, in different experiments, the identifiable involucrin-like sequences accounted for about 35% of the total retarded peptide material (molar amount) at the earlier times of proteolysis. These were attached through seven identifiable sequences of involucrin, most of which were located in the first 140 amino acid residues of the protein, in sequences thought to constitute the ancestral portion (30, 31). An exception is peptide 6, which is located in the more modern (still evolving) parts of the protein. Therefore, it could be argued that the attachment of ceramides to involucrin is an evolutionarily ancient aspect of barrier function in mammalian epidermis.

Identification of Other CE Proteins as Substrates of Lipid Attachment—Peaks 1, 2, 9, and 10, which were sensitive to the extent of proteolysis (Fig. 1), possessed complex amino acid compositions, suggesting the presence of multiple short peptide

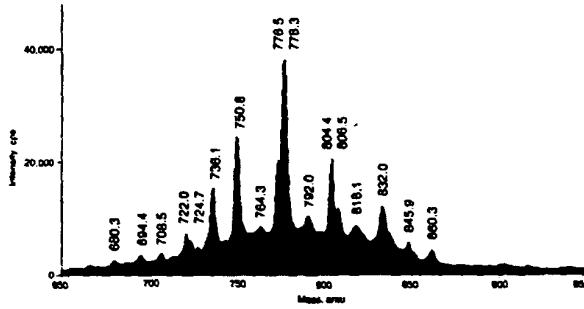


FIG. 5. Mass spectroscopy of ceramide lipids of pig skin. The data were collected as for Fig. 4 using a sample kindly supplied by Dr. Philip Wertz.

species. The material of peaks 9 and 10 accounted for <10% of the retarded material at the earliest time of digestion and was not explored further. Next, we pooled material from the mobile peaks 1 and 2 from the 10-, 20-, and 30-min digests, which accounted for about 50% (molar basis of peptide material) of the total retarded material in separate experiments (Fig. 1). Because these were poorly resolved by the C₄ HPLC column, we removed the ceramide adducts by saponification for 2 h and rechromatographed the released peptide material on a C₁₈ HPLC column (Fig. 3). Of this, 65–70% eluted at <8 min and could not be further resolved because it consisted of single amino acids or di- or tripeptides. Several peaks resolved at >10 min (~30% of total) were recovered for amino acid sequencing (Table II). Four matched with various nonhuman proteins, but

* L. N. Marekov and P. M. Steinert, unpublished observations.

TABLE IV
Likely composition of ceramides in human foreskin CEs

Major components (>7%) are shown in boldface.

Identified mass	Foreskin CEs, relative amount ^a	Pig skin, relative amount		Likely composition
		Measured	Expected ^b	
<i>atomic mass units</i>				
708	1.8	0.8	1	28:0/17:1
722	3.0	3.1	1	30:0/16:1
724	4.4	2.7	2	30:0/16:0
736	8.5	7.7	8	30:0/17:1
750	16.7	14.8	18	30:0/18:1
764	1.8	1.0	1	30:0/19:0
776	12.0	12.1	13	32:1/18:1
778	18.3	23.6	22	30:0/20:1
792	1.2	2.2	2	31:0/20:1
804	12.1	12.9	16	32:1/20:1
806	4.1	6.7	6	32:1/20:0
818	1.9	2.8	2	32:1/21:1
820	1.4	ND ^c	<1	32:0/21:1
832	7.4	7.5	6	34:1/20:1
834	1.8	ND	1	32:0/22:1
858	0.6	ND	<1	34:2/22:1
860	1.3	1.7	1	34:1/22:1
862	0.6	ND	<1	34:1/22:0

^a Calculated from the amount of each ceramide species multiplied by the amount of each peak of Fig. 1.^b Calculated from the relative amounts of each ceramide present in human (34, 35) and pig (36) skin.^c ND, not detected.

two coincided exactly with the novel CE protein periplakin.^{3,4} Two others were highly homologous to envoplakin (32) and periplakin by having a Glu to Gln residue substitution as seen for involucrin. Another peptide may have derived from envoplakin or desmoplakin (33). Each of these is a structural protein located at the cell periphery of keratinocytes, and they either have been established as (desmoplakin and envoplakin; Refs. 11–13) or are thought to be (periplakin) protein components of the CE. These data suggest that periplakin and envoplakin may each constitute 6–10% of the substrates for ceramide attachment. The other six retarded 2–4-residue peptides (about 6% of the total) commonly occur in these CE proteins, as well as in loricrin or involucrin (Table II). The remainder of the retarded material was composed of numerous other peptides of low abundances (Fig. 3; Table II).

Mass Spectroscopic Analyses of Ceramide-Peptide Adducts — Aliquots of 5–10 pmol of each ceramide-peptide peak from Fig. 1 were used for mass spectroscopic analyses. Each generated complex profiles with multiple mass species, of which only the regions containing the quantitatively major species are shown in Fig. 4 (left). Peaks 1, 2, 9, and 10 (data for peak 9 not shown) yielded profiles with broad peaks to which masses could not be assigned, presumably because of the presence of multiple short peptides (Tables I and II). In the cases of peaks 1 and 2, there also were minor mass components in the range of 1250–1750 atomic mass units (data not shown). However, peaks 3–8 all yielded profiles with species of well defined masses. Some peaks contained multiple species with masses differing by 2–4 atomic mass units. Notably, many peaks were separated from each other by 12–17 atomic mass units, most commonly 14 atomic mass units, suggestive of species differing by units of a methylene (CH_2) group and perhaps of species differing by double bonds and/or hydroxyl groups. The most complex profile was generated for peak 8, which consisted of two similar sets of species.

Next, we treated each peak by saponification for 2 h to hydrolyze the ester linkage between the ceramide and peptide moieties and repeated the mass measurements (Fig. 4, right). In all cases, we obtained well defined mass species ranging

from 664 to 888 atomic mass units, including peaks 1, 2, 9, and 10. Many of the peaks were also separated by an average of 14 atomic mass units. In each of the cases of peaks 3–8, an additional major species, representing approximately one-half of the total mass present in the samples was obtained that corresponded very closely (± 1 atomic mass unit) with the expected mass of the peptide as deduced from the amino acid sequencing analyses (Table I). Peak 8 material generated a simpler profile after saponification, and it generated two peptide peaks, establishing the presence of two ceramide-peptide adducts as implicated from the sequencing data (Table I). In the case of peaks 1, 2, 9, and 10 (data for peak 9 not shown), most of the poorly resolved ceramide-peptide adducts (Fig. 4, left) also contained a limited array of ceramides of well defined masses after saponification (right). Thus, these ceramides had been attached to a complex array of single amino acids or small peptides that themselves could not be resolved in this study by chromatography (Fig. 3 and Table II) or mass spectroscopy. However, in the cases of peaks 1 and 2, peptides of 887 and 962 atomic mass units were present (Fig. 4), which correspond to the masses expected for the peptides of sequence EQQLLQQ and EQEEAEAR and are probably derived from envoplakin and periplakin, respectively, recovered as described in Fig. 3 and Table II. Similarly, minor peaks of masses 702 and 736 atomic mass units were recovered (Fig. 4A, arrows) that correspond to two other peptides of sequences EELEAL and EQQTLQ, respectively (Table II). Peaks 6 and 10 contained other species (Fig. 4B, asterisks) that were also about 14 atomic mass units apart but were 5–7 atomic mass units different in size from the major ceramides. Because they were minor components, it is not known whether they are ceramides or some other lipid material that was ester-linked to the peptides.

Interestingly, there was a trend of increasing size of ceramide species from peak 1 to peak 10 (Fig. 4 and Table III), which is consistent with the notion that the later peaks were more retarded by the C_4 HPLC column because of the presence of ceramides having somewhat longer hydrophobic chains. A striking observation from the present work was that ceramide species of the same masses were present in several of the 10 peaks (Fig. 4 and Table III), including in particular masses of 750, 776, 778, and 804 atomic mass units. These data suggest that there were common higher abundance ceramides that

^a F. Watt personal communication.^b I. McLean and S. Aho, personal communication.

were used for attachment to multiple sites on involucrin and the other CE proteins.

Unfortunately, because only limited amounts of newborn foreskin epidermis was available to us, we were unable to acquire sufficient material to perform additional analyses to ascertain the exact fatty acid and sphingosine composition of the ceramides identified in this study. Nevertheless, we have performed two additional analyses. In the first, we determined the masses of ceramides recovered from pig skin (Fig. 5). The data show the presence of many ceramide species, and interestingly, components of masses of about 736, 750, 776, 778, 804, and 832 atomic mass units accounted for the majority. The chemical natures of the ω -hydroxy fatty acids and sphingosines of these ceramides and their relative abundances in both human and pig skin have been determined (34–36) and are listed in Table IV. In a second analysis, we calculated the relative amounts of the different ceramide masses identified in this study, based on the amount of each peak of Fig. 1 and the masses of the array of ceramides identified within each peak. Together, the comparisons of the pig and human data reveal remarkable consistency in especially the most abundant ceramides of masses 736, 750, 776, 778, 804, and 832 atomic mass units. These analyses thereby provide robust indirect data on the likely chemistry of the ω -hydroxy fatty acid and sphingosine components of the human ceramides recovered in the present work.

Conclusions—We have demonstrated that a family of ceramides within the range of 680–880 atomic mass units having fatty acid and sphingosine chains of varying size are covalently attached by way of ester bonds to involucrin as well as other known proteins of the human foreskin epidermal CE. This report confirms earlier work indicating that ceramides of this size range are esterified to proteins of human cornified epidermis (34, 35). Moreover, our work identifies for the first time some of the protein targets and residue positions of this attachment. However, the present experiments can account for only about 20% of the total ceramides of human foreskin epidermal CEs. Nevertheless, the analyses of Table III suggest that the recovered ceramides are representative of the total. On the other hand, we cannot exclude the possibility that a significant proportion of the ceramides are attached to other proteins of the CE.

Furthermore, these data are consistent with current models of the mechanism of assembly of the CE (7, 8, 10–13). Involucrin, desmoplakin, envoplakin, and periplakin have been identified as some of the earliest components assembled by transglutaminase cross-linking onto the protein CE (11–14) and are presumably located physically very close to the plasma membrane, near where keratin intermediate filaments interact in desmosomes. This complex is thought to function as a scaffold to which large amounts of loricrin and small proline-rich proteins are attached later on the cytoplasmic side. At some advanced stage of CE assembly, the plasma membrane is dispersed, leaving a cross-linked layer of protein on the exterior of the cell (7–13). Subsequently or concurrently, attachment of ceramides to these proteins is thought to occur from the external surface of the corneocyte (1–4, 18–23). Thus, the identification of involucrin, periplakin, and envoplakin as at least three of the protein targets of ceramide attachment is spatially and temporally consistent with existing data and models.

In addition, these data offer significant support to an earlier hypothesis that predicted that ceramides might be linked to a Glu/Gln-rich protein target (20, 23). Human involucrin contains about 40% of these residues (29). The other peptides

described here likewise resided in Glu/Gln-rich regions of the proteins (Tables I and II).

Future experiments now will focus on the mechanisms by which ceramides are delivered and become attached to the CE proteins. The linkage of the ceramides to Glu residues by simple ester bonds could involve acyl transferases. The linkage to Gln residues potentially may involve transglutaminases because they are capable of transferring an activated protein-bound Gln side chain to an alcohol acceptor, resulting in an ester bond (37).

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